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Efficacy of Fosfomycin Compared to Vancomycin in Treatment of Implant-Associated Chronic Methicillin-Resistant *Staphylococcus aureus* Osteomyelitis in Rats

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Fosfomycin monotherapy was compared to therapy with vancomycin for the treatment of implant-associated methicillin-resistant *Staphylococcus aureus* (MRSA) osteomyelitis in an experimental rat model. The proximal tibiae were inoculated with 15 μl of a suspension containing 1×10^8 to 5×10^8 CFU/ml of a clinical isolate of MRSA with simultaneous insertion of a titanium wire. Four weeks later, treatment was started for 28 days with either 50 mg/kg of body weight vancomycin intraperitoneally twice daily ($n = 11$) or 75 mg/kg fosfomycin intraperitoneally once daily ($n = 10$). Eleven animals were left untreated. After treatment, quantitative cultures from bone were found to be positive for MRSA in all animals in the untreated group (median, 3.29×10^6 CFU/g of bone) and the vancomycin group (median, 3.03×10^5 CFU/g of bone). In the fosfomycin group, MRSA was detectable in 2 out of 10 (20%) animals (3.42×10^2 and 1.51×10^3 CFU/g of bone). Vancomycin was superior to the no-drug control ($P = 0.002$), and fosfomycin was superior to the no-drug control and vancomycin ($P < 0.001$). The cultures from the wires were positive in all untreated animals (median, 2.5×10^3 CFU/implant), in 10 animals in the vancomycin group (median, 1.15×10^3 CFU/implant), and negative in all animals in the fosfomycin group. Based on the bacterial counts from the implants, vancomycin was not superior to the no-drug control ($P = 0.324$), and fosfomycin was superior to the no-drug control and vancomycin ($P < 0.001$). No emergence of resistance was observed. In conclusion, it was demonstrated that fosfomycin monotherapy is highly effective for the treatment of experimental implant-associated MRSA osteomyelitis.

Implant-associated osteomyelitis is considered to be a disastrous complication of orthopedic surgery associated with prolonged hospitalization, side effects of antibiotic therapy, and high economic costs (1). The current management of these forms of osteomyelitis involves implant-preserving treatment as well as one-step and two-step exchanges (2). All procedures, however, require long-term antimicrobial therapy lasting several weeks to months. Therefore, effective concentrations of the antimicrobial agent at the tissue surrounding the implant are of crucial importance, because bacteria adhere to the implant surface and may then reorganize and form a biofilm. More specifically, the bacteria produce an extracellular polymer-rich matrix that provides protection against antibiotics and macrophages (1).

Staphylococcus aureus is one of the most common pathogens causing periprosthetic infections. The rising incidence of infections with multidrug-resistant bacteria, like methicillin-resistant *S. aureus* (MRSA), however, limits the antimicrobial treatment options available. Thus, there is a critical need for antimicrobial treatment regimens involving new or old antibiotics with activities against adherent and metabolically less active bacteria and a favorable side effect profile for long-term therapy against bacteria with multiple drug resistance profiles (2).

Fosfomycin (FOF) is a well-tolerated bactericidal agent with long-standing sensible clinical use. FOF displays broad-spectrum activity against various Gram-positive and distinct Gram-negative bacteria, including difficult-to-treat pathogens, such as MRSA, daptomycin-resistant *S. aureus*, penicillin-resistant pneumococci, and extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* (3). FOF penetrates well into osseous tissue and has proved to be clinically useful for the treatment of acute and chronic osteomyelitis (3, 4). In the clinical routine, however, FOF is mainly used in combination with other classes of antibiotics because of the synergism frequently observed and the concern about the development of resistance (5). However, the clinical relevance of developing resistance has been recently questioned (6, 7). Further, recent data from preclinical studies have shown that FOF is effective for the treatment of biofilm-associated infections when combined with other classes of antibiotics (8–10). However, data on the efficacy of FOF monotherapy for the treatment of implant-associated infections in osseous tissue are limited. The aim of the present study was to evaluate the efficacy of

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such treatment in comparison to a treatment with vancomycin (VAN) in a controlled animal study using an experimental rat model of chronic implant-associated MRSA osteomyelitis.

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MATERIALS AND METHODS

The study protocol was approved by the local animal welfare committee and was performed in the level 2 biohazard area of the Institute of Biomedical Research, Medical University of Vienna, Vienna, Austria.

Bacteria and preparation of inocula. The pathogen used was a clinical strain of MRSA (4409/07) isolated from a patient with chronic osteomyelitis. For *in vivo* testing, bacteria in the exponential growth phase were used as follows. Bacteria grown overnight in Trypticase soy broth (TSB) were diluted 1:100 in TSB and incubated for 5 h at 37°C. A bacterial inoculum containing 1×10^8 to 5×10^8 CFU/ml was prepared, and the number of viable organisms was retrospectively confirmed from the plate counts made before and after the surgical procedure.

***In vitro* susceptibility testing.** The MIC of FOF was determined using the broth microdilution method in cation-adjusted Mueller-Hinton broth (CAMHB) supplemented with glucose-6-phosphate (Sigma-Aldrich) at a final concentration of 25 mg/liter (12). The MIC of VAN was determined by using the epsilometer test (Etest) method.

Experimental osteomyelitis. Implant-associated osteomyelitis was established using a modification of Zak's model of experimental osteomyelitis described by Vergidis et al. (13). In this animal model, instead of the sclerosing agent morrhuate sodium, a titanium wire is implanted to promote the onset of infection. In total, 40 male Sprague-Dawley CD rats (Charles River WIGA GmbH, Sulzfeld, Germany), weighing 350 to 400 g each, were used. Each animal was anesthetized with ketamine and xylazine, and the left hind leg was shaved and disinfected with polyvinyl pyrrolidine-iodine. The proximal medial surface of the tibia was surgically exposed, and a hole (0.1-cm diameter) was made in the medullary cavity using a high-speed drill (MultiPro; Dremel, Racine, WI). A 15-μl sample of the MRSA inoculum containing 1×10^8 to 5×10^8 CFU/ml was injected into the bone, followed by the insertion of a 10 mm by 0.8 mm titanium Kirschner wire. The drill holes were sealed with sterile bone wax (Ethicon Sutures, Ltd., Peterborough, Ontario, Canada) to avoid leakage of the inoculum, and the incisions were closed with sutures.

Histopathology. For histological confirmation of chronic implant-associated osteomyelitis, bone specimens from one uninfected and three infected animals were analyzed 4 weeks after establishing infection. The rat tibiae containing the implants were fixed in 4% neutral buffered formalin for a week. After rinsing them with tap water to remove the formalin, the specimens were dehydrated in ascending grades of alcohol. The next steps were infiltration with and finally embedding in a light-curing resin (Technovit 7200; Kulzer & Co., Wehrheim, Germany). Using the cutting-grinding technique of Donath (14), undecalcified thin-ground sections of approximately 30 μm in thickness were prepared and stained with Levai Laczko dye. For each rat tibia, one ground section was prepared in the dorsoventral direction along the implant axis. The cutting-grinding equipment used was produced by Exakt (Norderstedt, Germany). The slices were scanned using the Olympus DotSlide 2.4 digital virtual microscopy system (Olympus, Tokyo, Japan). Images with a resolution of 0.32 μm per pixel were prepared.

Antimicrobial treatment regimens. Four weeks after infection was established, animals with radiographically confirmed implant-associated osteomyelitis of the tibia were arbitrarily assigned to receive either FOF ($n = 10$) or VAN ($n = 11$). Eleven animals were left untreated.

FOF powder (Sandoz, Kundl, Austria) was dissolved in sterile water and administered intraperitoneally at a dose of 75 mg/kg of body weight (BW) once daily. VAN powder (Xellia Pharmaceuticals ApS, Copenhagen, Denmark) was dissolved in sterile water and administered intraperitoneally at a dose of 50 mg/kg of BW twice daily.

Bacterial counting. Sixteen to 20 h after the last administration of antibiotics, each animal was euthanized with a lethal dose of thiopental, and the infected tibiae were aseptically removed. After explantation of the implant, the bones were weighed and then pulverized with a CryoMill. Sterile physiological saline (10 ml) was added to each specimen, and the bone suspensions were vigorously vortexed. The bone suspensions prepared from the infected tibiae were serially diluted and plated on sheep blood agar plates. Quantitative culture results for osseous tissue were obtained after 48 h of incubation and expressed as CFU/g of bone. The limit of detection for viable counts was 50 CFU/g of bone. To detect bacteria that adhered to implants, Kirschner wires were put into 2 ml sterile physiological saline, vortexed for 30 s, sonicated at 35 kHz for 5 min, and vortexed again for 30 s. Quantitative cultures were performed as described above. For qualitative cultures, 500 μl of the remaining 2 ml saline was plated on sheep blood agar plates, and the Kirschner wires were rolled and placed on sheep blood agar plates. The limit of detection for viable counts from Kirschner wires was 4 CFU/implant.

To detect the emergence of resistance, the MICs of FOF and VAN for MRSA recovered from bones and implants were determined using the standard methods described above.

FOF concentrations in bone. The FOF concentrations were determined in infected and healthy tibiae at the end of the study, 16 to 20 h after the last administration of FOF. Therefore, the uninfected tibiae from all animals in the FOF group were processed as described above. To increase the extraction of FOF from osseous tissue, all bone suspensions were centrifuged and rediluted with 10 ml sterile physiological saline, vigorously vortexed again, and sonicated at 35 kHz for 5 min. This procedure was repeated two times. The FOF concentrations in the bone suspensions were determined in duplicate using liquid chromatography/tandem mass spectrometry (LC-MS/MS), as described elsewhere (15).

Statistics. The data are given as the mean ± standard deviation or as median values, as appropriate. Pairwise comparisons between the bacterial counts of the three treatment arms were performed using the Mann-Whitney U test, followed by Bonferroni's correction for multiple comparisons. Therefore, P values of <0.016 were considered statistically significant to adjust for three pairwise comparisons. To compare the FOF concentrations in the healthy and infected bones, a t test was used, and a P value of <0.05 was considered statistically significant. All results were plotted and analyzed using GraphPad Prism version 5.02 (GraphPad Software, Inc., CA).

RESULTS

Experimental osteomyelitis. Four out of 40 rats died during anesthesia and were not further analyzed. Four weeks after infection with MRSA, 35 of the 35 infected rats (100%) (one animal was left uninfected for histological evaluation) had radiographically confirmed localized implant-associated osteomyelitis of the tibia.

The histological examination of embedded specimens from three infected animals displayed numerous intramedullary neutrophilic granulocytes, edema, isolated microabscesses with colonies of cocci, and focal defects and resorption in the preexisting bone (Fig. 1). Partly woven bone was newly built around the metal shaft.

The results of antibiotic treatment in the experimental implant-associated MRSA osteomyelitis cases are shown in Fig. 2. Bacterial cultures from bone were found to be positive for methicillin-resistant *S. aureus* in 11 of 11 (100%) animals in the untreated group, 11 of 11 (100%) animals treated with VAN, and 2 of 10 (20%) of the FOF-treated animals.

Among the untreated animals, the median bacterial count (minimum to maximum) was 3.29×10^6 CFU/g of bone ($3.71 \times$

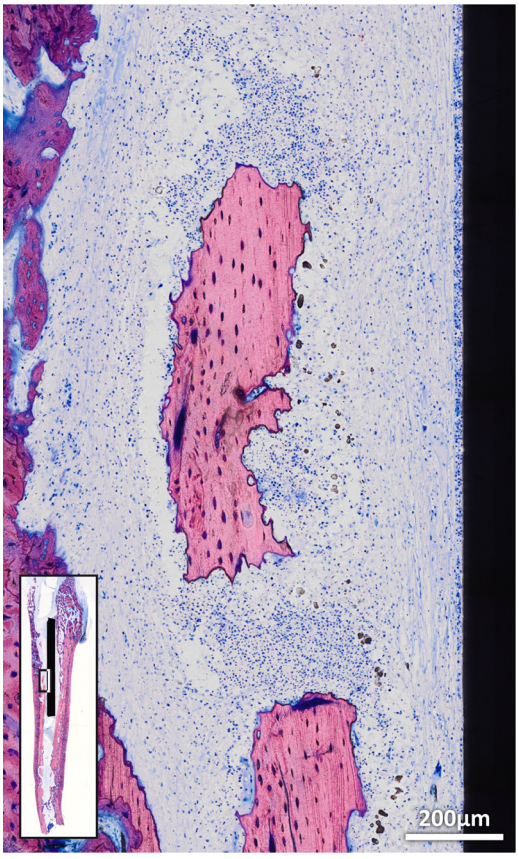

FIG 1 Histological characteristics. Shown are numerous intramedullary neutrophilic granulocytes, edema, isolated microabscesses with colonies of cocci around the metal shaft, and resorption on remnants of preexisting bone. (Inset) Newly built woven bone at a small distance from the metal shaft (Levai Laczko stain, original magnification, ×200).

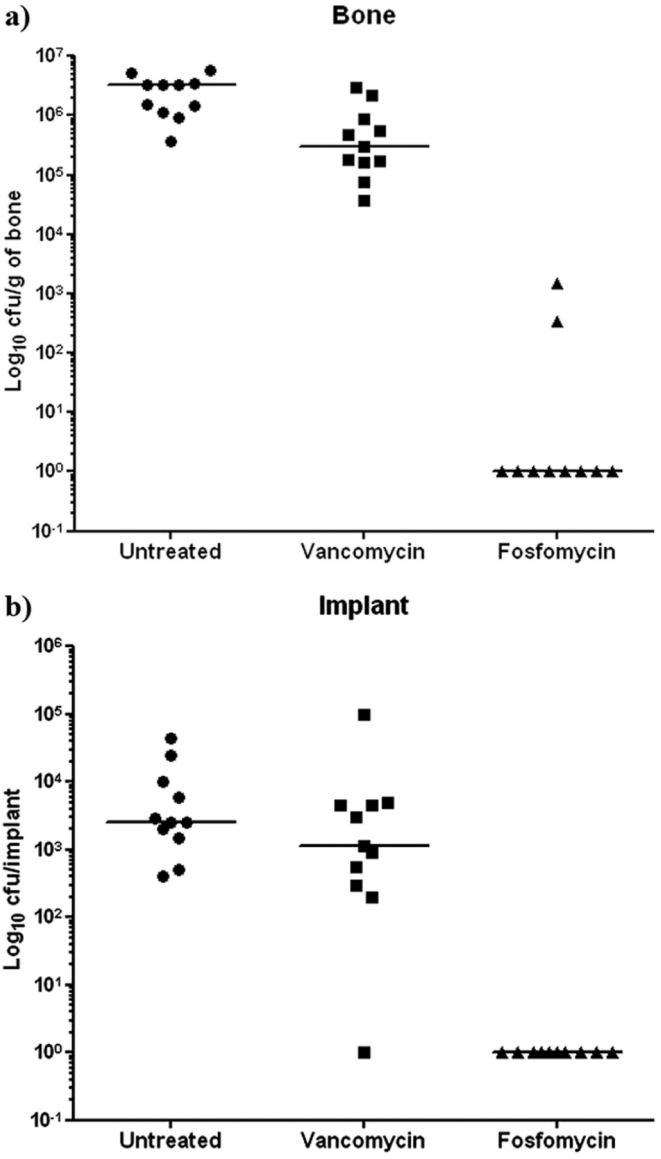

FIG 2 Bacterial counts in osseous tissue (a) and on implants (b) after a 4-week treatment period with fosfomycin or vancomycin in experimental implant-associated MRSA osteomyelitis model in rats. The groups consisted of untreated ($n = 11$), vancomycin-treated ($n = 11$), and fosfomycin-treated ($n = 10$) rats. Each symbol represents the value for an individual animal; horizontal bars indicate median values.

10^5 to 5.73×10^6). Treatment with VAN resulted in a median bacterial count of 3.03×10^5 CFU/g of bone (3.67×10^4 to 2.92×10^6) and was significantly superior to the count for the no-drug control ($P = 0.002$). The bacterial counts in the two animals with detectable bacteria in the FOF group were 3.42×10^2 and 1.51×10^3 CFU/g of bone. The results of treatment with FOF at one dose of 75 mg/kg of body weight were thus significantly superior to the results in the no-drug control ($P < 0.001$) and the VAN group ($P < 0.001$).

The cultures from the Kirschner wires were positive in all animals in the untreated group, 10 out of 11 animals in the VAN group, and negative in all animals in the FOF group. The median bacterial counts derived from the Kirschner wires were 2.5×10^3 CFU/implant (4.0×10^2 to 4.5×10^4) in untreated animals and 1.15×10^3 CFU/implant (0 to 1×10^5) in the VAN group. Based on the bacterial counts derived from the implants, VAN was not significantly superior to no treatment ($P = 0.324$), but FOF was significantly superior to no treatment ($P < 0.001$) and VAN ($P < 0.001$). Both study drugs were well tolerated. None of the animals developed diarrhea, and between the three treatment arms, no differences in body weight were observed at the end of the study.

FOF concentrations in bone. The mean concentrations of FOF in the bones harvested 16 to 20 h after the last administration were significantly higher in the infected tibiae (mean ± standard deviation [SD], 0.85 ± 0.2 μg/g of bone) than the concentrations found in uninfected tibiae (mean ± SD, 0.62 ± 0.15 μg/g of bone) ($P = 0.01$). The mean ± SD concentrations of FOF in the first suspension of the three bone extraction suspensions prepared, which was used for bacterial culture, were 0.1 ± 0.02 μg/ml for suspensions derived from infected bones and 0.07 ± 0.03 μg/ml for suspensions derived from uninfected bones.

MICs and development of drug resistance. The MICs of FOF and VAN against MRSA 4409/07 were 0.25 μg/ml and 1.5 μg/ml, respectively. The MICs of isolates of MRSA obtained from all animals still infected at the end of the 4-week treatment period were within one dilution of the pretreatment MIC, indicating no emergence of resistance.

DISCUSSION

In recent years, FOF has increasingly gained attention in the treatment of biofilm-associated infections. Experimental studies in rats with biofilms induced by *S. aureus* and *Pseudomonas aeruginosa* have shown that FOF induces changes in biofilm structure and the inflammatory response (9, 16, 17). Further, it was shown that FOF increases the penetration of concomitant antimicrobial agents into biofilms, resulting in increased eradication of embedded bacteria (9, 16–18). Likewise, an *in vitro* study very recently showed that FOF enhances the activities of linezolid, minocycline, vancomycin, and teicoplanin against planktonic or biofilm-embedded MRSA organisms (10). These combinatorial treatments were even better than rifampin combination regimens and indicate the therapeutic advantages for catheter-associated or prosthetic joint infections (10).

In humans, FOF has been shown to be effective when combined with daptomycin in patients with endocarditis (19), as well as in combination with cefotaxime in patients with meningitis, septicemia, and bone and joint infections (20). Thus, clinical experience with FOF is mainly limited to regimens that combine FOF with other antibiotics.

In the present study, however, it was demonstrated that FOF is effective in the treatment of experimental implant-associated MRSA osteomyelitis when used as a monotherapy. This is in contrast to findings by Garrigós et al. (21), who found a low efficacy for FOF monotherapy in the treatment of biofilm-associated infections caused by MRSA in a 72-h cage rat tissue infection model. The treatment period was 7 days.

Thus, the high efficacy of FOF monotherapy found in the present study appears surprising. One possible explanation for this unexpected finding might be the longer treatment period of 4 weeks applied in the present study, which is, however, more likely to reflect the clinical situation when treating implant-associated osteomyelitis.

However, in humans, the dosing regimens of FOF range from 3 g daily for uncomplicated urinary tract infections to 8 g three times daily for severe staphylococcal infections or infections with Gram-negative bacteria (3). It was recently shown that in laboratory rats, a dosing regimen of 500 mg/kg three times daily results in an AUC of FOF corresponding to an AUC in humans when used at a dosage of 8 g three times daily (15). Although the pharmacokinetics of fosfomycin at a steady state was not investigated in that study, the concentration-over-time profile indicates that no accumulation of fosfomycin after repeated administration is to be expected in rats, due to the short half-life ($t_{1/2}$) observed (15).

In the present study, a dose of 75 mg/kg is a significantly lower dosage than is usually applied. As previously shown, 75 mg/kg of body weight in the rat corresponds to drug levels considerably below those achieved after all currently employed dosing regimens applied in humans. Therefore, we consider our model using the dosage of 75 mg/kg body weight a very conservative estimate.

Another explanation for the high efficacy might be that FOF penetrates osseous tissue well and displays osseophilic behavior that is attributed to its structural similarity to hydroxyapatite (4). This is also supported by the fact that FOF was still detectable in osseous tissue 16 to 20 h after the last administration in the present study. Considering the short $t_{1/2}$ of only 0.7 h observed in rats (15), the present data strongly indicate that FOF accumulates in infected bone.

Based on CLSI criteria, the susceptibility of *S. aureus* strains to FOF includes MIC values of ≤16 mg/liter. It must be emphasized that the MIC of 0.25 mg/liter of the MRSA strain used in the present study is particularly low, and in infected osseous tissue, FOF concentrations 16 to 20 h after the last administration were still around 0.85 μg/g of bone. Thus, for other strains of *S. aureus* with higher MICs, a higher dosage of FOF might be necessary, and care should be taken when extrapolating the results of the present studies to infections with other staphylococcal strains.

Nonetheless, and in light of a presumed accumulation of FOF in osseous tissue, antibiotic carryover should also be discussed as an explanation for the high percentage of negative cultures observed in the FOF group. In the present study, bone samples were obtained 16 to 20 h after treatment cessation, which is a longer gap than that used in comparable studies (13, 22). Moreover, the residual FOF concentrations determined in the bone suspensions used for bacterial counting were found to be below the MIC of the MRSA strains used in the present study. Considering that quantitative bacterial counting was performed with serial 10-fold dilutions, antibiotic carryover should possibly be excluded as an explanation for negative culture results.

Although VAN is an accepted standard antibiotic used in the treatment of implant-associated infections, and it significantly reduced the number of bacteria in bone, the overall performance of VAN was poor compared with that of FOF. This is supported by data from other animal studies. Regarding implant-associated methicillin-susceptible *S. aureus* (MSSA) osteomyelitis in rats, no beneficial effect was observed for VAN when applied at a dosage of 15 mg/kg of body weight (BW) intraperitoneally (i.p.) twice daily over 21 days (23) or 50 mg/kg of BW i.p. twice daily over 21 days (13). Possible explanations for the low efficacy observed for VAN in the treatment of experimental implant-associated osteomyelitis might be poor penetration into osseous tissue and biofilms of this large molecule or the alteration of the antibiotic by biofilm products (24).

Of interest, after 4 weeks of administration of FOF or VAN as a monotherapy, no emergence of resistance was observed in any of the treatment groups. This is particularly surprising for FOF, which is usually used in combination because of a high rate of resistance observed *in vitro*. For MRSA isolates obtained from patients with cystic fibrosis, mutation frequencies of 1×10^{-5} to 1×10^{-8} were found (25).

In the present study, the mean number of CFU obtained from bones of the no-treatment group (not weight based) was 2.7×10^6. Thus, it cannot be excluded that the number of viable colonies was below the threshold of the mutation frequency at which resistance to fosfomycin occurs. On the other hand, recent studies have demonstrated that the development of chromosomal resistance to FOF entails a biological cost that reduces the virulence and biological fitness of pathogens (6, 7).

Further, it is reasonable to assume that the risk for antimicrobial resistance is related to the concentration at the target site and pharmacodynamic parameters of a particular antimicrobial agent (26). Considering that FOF displays time-dependent efficacy (27, 28), the absence of emerging FOF resistance might also be explained by the high concentrations found in osseous tissue, together with the particularly low MIC of the strain used. Thus, the absence of emerging resistance against FOF observed should be interpreted with regard to the particular conditions of the present study. One limitation of the present study is that no serum con-

centration-versus-time profile was determined. However, the serum pharmacokinetics of FOF and VAN are well described in the literature, and for VAN, dosages from 15 mg/kg to 50 mg/kg of body weight twice daily have been used in experimental osteomyelitis studies in rats (13, 23, 29). Thus, it is rather unlikely that an underdosage of VAN or overdosage of FOF is an alternative explanation for the results obtained in the present study.

In summary, even in a low-dose regimen, FOF was highly efficacious for the treatment of experimental implant-associated MRSA osteomyelitis in rats. After prolonged therapy, emerging resistance to FOF or VAN was not detected. Although the treatment of difficult-to-treat infections or biofilm-related infections with antimicrobial monotherapy must be undertaken with care because of the expected lower efficacy and the risk of resistance, the clinical effectiveness of FOF monotherapy in periprosthetic *S. aureus* infections should be further evaluated. Additional studies using representative strains and clinical representative dosing regimens should be performed for estimations of clinical efficacy of FOF monotherapy in humans.

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